EXHIBIT F

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Antibody Microarrays: Current Status and Key Technological Advances

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ABSTRACT

Antibody-based microarrays are among the novel classes of rapidly evolving proteomic technologies that holds great promise in biomedicine. Miniaturized microarrays (<1 cm^2) can be printed with thousands of individual antibodies carrying the desired specificities, and with biological sample (e.g., an entire proteome) added, virtually any specifically bound analytes can be detected. While consuming only minute amounts (<μL scale) of reagents, ultra-sensitive assays (zeptomol range) can readily be performed in a highly multiplexed manner. The microarray patterns generated can then be transformed into proteomic maps, or detailed molecular fingerprints, revealing the composition of the proteome. Thus, protein expression profiling and global proteome analysis using this tool will offer new opportunities for drug target and biomarker discovery, disease diagnostics, and insights into disease biology. Adopting the antibody microarray technology platform, several biomedical applications, ranging from focused assays to proteome-scale analysis will be rapidly emerging in the coming years. This review will discuss the current status of the antibody microarray technology focusing on recent technological advances and key issues in the process of evolving the methodology into a high-performing proteomic research tool.

INTRODUCTION

In recent years, the field of large-scale biology has made tremendous progress and provided us with golden opportunities to decipher and understand the function of complex biological networks. Several hundreds of genomes have been sequenced, including the human, providing us with the blue-prints for organisms paving the way for major breakthroughs in genomics (Hanash, 2003; Staudt, 2002). Fueled by these advances, proteomics—the large-scale analysis of proteins—has become a key discipline for mapping the protein expression patterns and for understanding the function and regulation of the entire set of proteins encoded by an organism (Hanash, 2003; Jang and Hanash, 2003; Phizicky et al., 2004; Yanagida, 2002; Zhu et al., 2003). This information will be essential for understanding complex biological processes at the molecular level, how they differ in various tissues and cell types, and how they are affected in disease states (Borrebaeck, 2006; Hanash, 2003).

The opportunities and challenges facing proteomic approaches are formidable. The impact of large-scale proteome analysis in biomedicine (disease proteomics) is already substantial and a feature that will continue to grow in coming years (Hanash, 2003; Phizicky et al., 2004; Zhu et al., 2003). Particularly promis-

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ing effects commonly identified include: a better understanding of disease processes, discovery of novel biomarkers for diagnosis and early detection, or even prevention, of disease, and accelerated development of new drugs (Borrebaeck, 2006; Hanash, 2003; Phizicky et al., 2004; Zhu et al., 2003). In this process, the proteome must be readily addressable, not only at the whole-cell or tissue levels, but also in sub-cellular structures, in various complexes and in biological fluids. However, the complexity and dynamic nature of a proteome represents a major technological challenge. Thus, the technology platforms applied must provide a multiplexed set-up that is able to capture this dynamic state in a specific and sensitive manner irrespective of sample origin and format. Tackling these challenges and the numerous facets of (disease) proteomics will require multiple strategies and technology platforms to be implemented (Hanash, 2003; Jang and Hanash, 2003; Zhu et al., 2003).

Emerging proteomic technologies: protein microarrays

A set of rapidly evolving key technologies have emerged making it, to various degrees of extent, possible to generate expression patterns of multiple proteins in semi-complex samples, to identify protein-protein interaction networks, and to analyse their biological functions (Macbeath, 2002; Phizicky et al., 2004; Wingren and Borrebaeck, 2004; Yanagida, 2002; Zhu et al., 2003). To date, most of these strategies have been directed towards soluble analytes, while there are very few effective approaches for profiling membrane proteomes (Jang and Hanash, 2003). Classical separation techniques, such as two-dimensional gels, combined with mass spectrometry (MS) or tandem-MS is a versatile proteomic research tool frequently used for protein expression profiling and identification of post-translational modifications (Adkins et al., 2003; Anderson et al., 2004; Hanash, 2003; Tirumalai et al., 2003; Yanagida, 2002; Zhu et al., 2003). Although this is a powerful and important technique for proteome analysis it still has methodological limitations, including: limited resolution (<1,000 protein analytes per sample), expensive, time-consuming, relatively low-throughput and technically demanding (Yanagida, 2002; Zhu et al., 2003). Among the newer methodologies implemented, the affinity protein microarray technology (Fig. 1) is among the most promising approaches (Borrebaeck, 2000; Haab, 2003; Macbeath, 2002; Poetz et al., 2004; Wilson and Nock, 2003; Wingren and Borrebaeck, 2004; Zhu and Snyder, 2003). To this end, a variety of biochips have been designed and tremendous efforts have been made to devise strategies for producing protein arrays that have direct utility for (bio)medical applications (Angenendt, 2005; Borrebaeck, 2006; Haab, 2003; Kingsmore, 2006; Macbeath, 2002; Poetz et al., 2004; Wilson and Nock, 2003; Wingren and Borrebaeck, 2004; Zhu and Snyder, 2003). This repertoire of (affinity) protein microarrays have already demonstrated that they will play an important role in filling the gap between genomics and proteomics by providing high-throughput and multiplexed assays in a highly specific and sensitive manner (Haab, 2003; Macbeath, 2002; Pavlickova et al., 2004; Poetz et al., 2004; Wingren and Borrebaeck, 2004, 2006; Zhu et al. 2001; Zhu and Snyder, 2003)

Antibody-based microarrays

Protein microarrays can be divided into two conceptual groups, functional protein microarrays (functional proteomics) and affinity protein microarrays (quantitative proteomics) (Fig. 1) (Macbeath, 2002; Poetz et al., 2004). Relying mainly on antibodies as content, affinity protein microarrays have been successfully used in proteomics, ranging from focused assays to large scale analysis (Angenendt, 2005; Borrebaeck, 2006; Haab, 2003; Kingsmore, 2006; Macbeath, 2002; Pavlickova et al., 2004; Wilson and Nock, 2003; Wingren and Borrebaeck, 2004; Zhu and Snyder, 2003). The technology involves the dispensing of minute volumes of individual antibodies, specific for the target analytes (e.g., proteins, haptens, and carbohydrates etc.), onto solid supports in μm to nm sized spots/wells (Fig. 1). The printed microarrays, ranging in density from a few single clones to thousands of antibody probes, are then incubated with the sample. Next, the specifically bound analytes are detected and the data generated transformed into a molecular map showing the detailed expression patterns of the individual analytes. Major efforts are currently under way to evolve the antibody microarray technology into the high-throughput proteomic research tool needed by the research community (Pavlickova et al. 2004; Wingren and Borrebaeck, 2004, 2006). In this process, several critical sub-areas have been identified (Wingren and Borrebaeck, 2006), including (i) solid support, (ii) content,

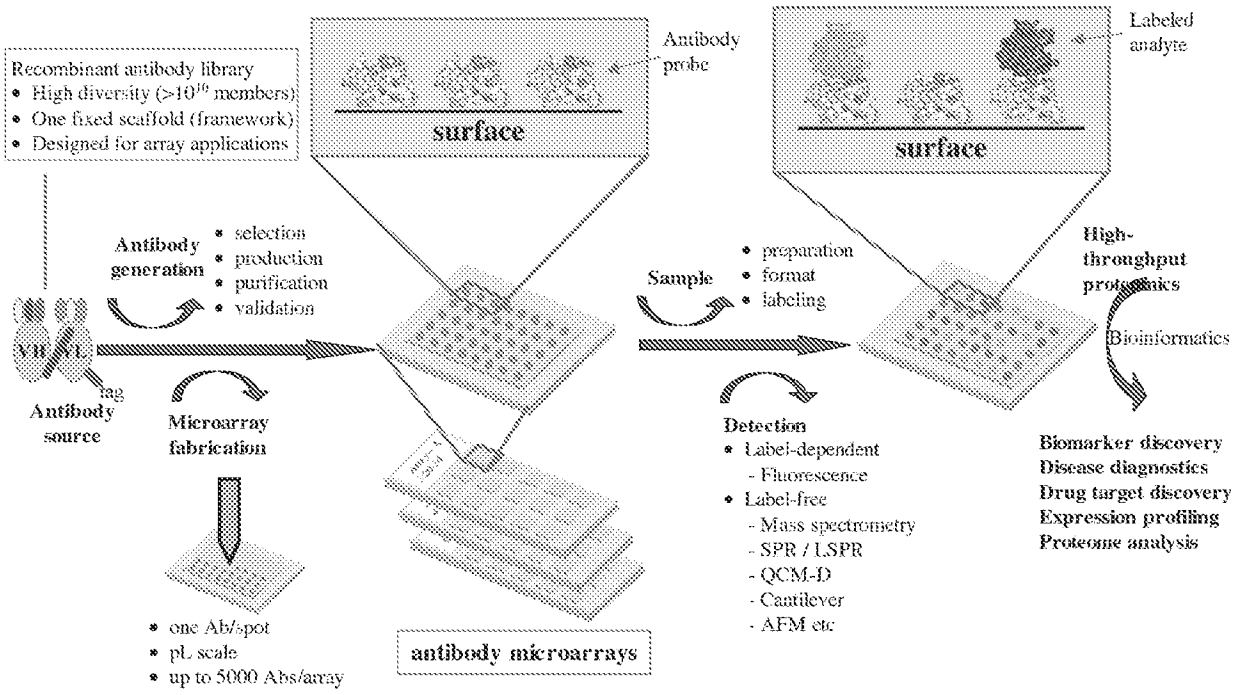

FIG. 1. Schematic illustration of the antibody microarray set-up.

(iii) array format/fabrication, (iv) sample, (v) analytical principle, (vi) standardization and benchmarking, and (vii) data processing. This review discusses the current status of the antibody microarray technology platform focusing on recent technological advances and key issues remaining to be resolved. Recent applications based on the antibody microarray technology have been described (Gao et al., 2005; Knezevic et al., 2001; Miller et al., 2003; Orchekowski et al., 2005; Sanchez-Carbayo et al., 2006; Sreekumar et al., 2001) and reviewed (Borrebaeck, 2006; Kingsmore, 2006; Wingren and Borrebaeck, 2004) elsewhere and will not be discussed.

SOLID SUPPORTS

In the quest of designing high-performing antibody microarrays, the design of the solid support will play an essential role (Angenendt et al., 2002, 2003; Kusnezow and Hoheisel, 2003). Both the immobilization of the protein content as well as the subsequent binding of targeted protein analytes can be affected. First, proteins are sensitive to the physical and chemical properties of the supports, and any given surface will not be suitable for all proteins. Second, the deposition of protein probes may result in partial, or complete, denaturation, which can impair the subsequent probe-ligand interaction. To date, a large repertoire of solid supports based on predominantly glass-, plastic-, or silicon-slides, often modified with one-, two-, or three-dimensional structured surface modifications (e.g., nitrocellulose) have been fabricated (Angenendt et al., 2002, 2003; Beator, 2002; Guilleaume et al., 2005; Kusnezow and Hoheisel, 2003; Ressine et al., 2003; Rubina et al., 2003; Steinhauer et al., 2005; Stillman and Tonkinson, 2000; Wang et al., 2002; Wilson and Nock, 2003; www.arrayit.com; www.nuncbrand.com; www.perkinelmer.com; www.whatman.com; www.schott.com; www.xenopore.com). The precise choice of substrate is, however, not obvious and will be dependent on several factors such as the probe source (e.g., purified vs. none-purified, and species/format), the coupling chemistry required (e.g., adsorption, covalent coupling or affinity binding), the sample complexity (e.g., pure vs. complex), and the sensitivity desired. Four key properties of the substrate to consider in this selection procedure are (i) high biocompatibility, (ii) high and selective probe binding capac-

ity, (iii) ability to bind the probes in a favorable orientation, and (iv) low non-specific (background) binding.

During recent years, a conception that surface engineering is no longer a high priority area has surfaced, since adequate designs are already at hand. But despite the selection of advanced and improved surface designs available, we strongly believe that a significant focus on substrate engineering should be maintained. In fact, a selection of substrates, or even a single support, capable of meeting all, or most, of the essential criteria listed above still remains to be fabricated.

Recent advances

Antibody arrays fabricated by deposition of individual probes onto planar surfaces, in the format of either microscope slides (25 × 75 mm) or well-defined wells (e.g., ELISA plate), are the most commonly used designs. In terms of protein arrays in general, alternative architectures are available, including for example vial-based surfaces significantly reducing the reaction volumes (Angenendt et al., 2005; Ghatnekar-Nilsson et al., 2006), individual 3D silicon-based posts (www.zyomyx.com) and compact devices combined with advanced microfluidics (www.gyros.com). In addition, recent advances have highlighted that arrays based on particles (beads) in solution may also be an interesting approach, provided that they are coded for identification (Poetz et al., 2004; Szodoray et al., 2004; Warren et al., 2004). Current systems include colour coding for microbeads (www.biorad.com; www.luminexcorp.com), semiconductor crystals (www.qdots.com), barcoding for beads (www.smartbead.com) and multimetal microrods (www.nanoplextech.com). Interestingly, beads can also be assembled into planar arrays on semiconductor chips (www.bioarrays.com).

Novel high-performing physical supports include macro- and nano-porous silicon, non-modified (Ressine et al., 2003, 2005) or coated with nitrocellulose (Steinhauer et al., 2005) (high probe binding capacity and high biocompatibility), providing a platform compatible with both fluorescence- and MS-based detection (Finnskog et al., 2004; Ressine, 2003; Steinhauer et al., 2005). Further, a novel supramolecular hydrogel has been designed that may allow minute amounts of water to be trapped within the three-dimensional structure of the substrate providing a semi-moist environment, appropriate for protein arrays (Kiyonaka et al., 2004). In addition, recent work has indicated that novel commercially available substrates, including for instance Nexterion slide H (www.schott.com) (high signal intensities), protein-binding glass slides (low background, high biocompatibility) (www.nuncbrand.com) and black polymer MaxiSorp microarray slides (low background, high biocompatibility, high sensitivity) (www.nuncbrand.com) have turned out to be particular promising for recombinant antibody arrays. In particular, the black MaxiSorp slides have demonstrated excellent performances even when targeting directly labeled complex proteomes (Ingvarsson et al., 2006a,b; Wingren et al., 2006).

To date, a limited selection of functionalized substrates allowing affinity binding of the arrayed affinity-tagged probes have been designed, including Ni^{2+}-slides (Svedhem et al., 2003; www.xenopore.com), streptavidin-coated slides (e.g., www.xenopore.com), biotin-coated substrates (e.g., www.zeposens.com; www.zyomyx.com), and DNA modified slides (Choi et al., 2005; Svedhem et al., 2003; Wacker and Niemeyer, 2004; Wacker et al., 2004). This is an attractive set-up that will enable crude probe preparations to be purified, coupled, enriched and specifically orientated in a one-step procedure directly on the chip (affinity-on-a-chip) at high density. Recent work has shown that the performance of antibody microarrays fabricated by affinity directed immobilization vs direct spotting (adsorption or covalent coupling) in many cases are superior (Pavlickova et al. 2003; Peluso et al., 2003; Ruiz-Taylor et al., 2001; Wacker and Niemeyer, 2004; Wacker et al., 2004; Zhu et al., 2001). Among the most prominent features observed were better spot morphologies, enhanced experimental reproducibility, significantly reduced (at least 100–fold less) amount of probe required, increased functionality and improved sensitivity (Pavlickova et al. 2003; Peluso et al., 2003; Ruiz-Taylor et al., 2001; Wacker and Niemeyer, 2004; Wacker et al., 2004; Zhu et al., 2001). Of note, recent work has shown that photonic activation of disulfide bridges can be applied to achieve oriented and spatially covalent coupling of the probes, such as Fab molecules, onto thiol-reactive surfaces (Neves-Petersen et al., 2006). Further, affinity-based immobilization, such as DNA-directed coupling, will ultimately allow self-adressable arrays to be fabricated (i.e., arrays to which probes can be added in bulk and the probes will then find the way to their unique spot on their own) (Ramachandran et al., 2004; Wacker and Niemeyer, 2004; Wacker et al., 2004). Future efforts directed towards increasing the quality as well as

the selection of functionalized substrates will thus play a key role in the development of the next generation of high-performing antibody microarrays.

ANTIBODY PROBES

Since the introduction of the first affinity protein microarrays, antibodies have constituted the obvious choice of content, since they are by far the most well-documented binding molecules (Macbeath, 2002; Pavlickova et al., 2004; Wilson and Nock, 2003; Wingren and Borrebaeck, 2004; Zhu and Snyder, 2003). In recent years, a variety of antibody mimics, such as Affibodies (a three-helix bundle based on the scaffold of one of the IgG-binding domains of Protein A) (Renberg et al., 2005; www.affibody.com), trinectin binding proteins (a scaffold adapted from fibronectin; www.phylos.com), ankyrins (proteins based on stacked, 33–amino acid repeats) (Binz et al., 2004), and aptamers (short protein binding oligonucleotides) (Bock et al., 2004; www.archemix.com; www.somalogic.com), have been designed and evaluated as potential capture agents. The rational behind selecting antibodies over any of these various antibody mimics as content have recently been reviewed elsewhere (Pavlickova et al., 2004,; Wingren and Borrebaeck, 2004) and will not be discussed further.

Recent advances

The first generation of antibody-based microarrays was successfully generated using mainly readily available (off-the-shelf) purified, intact monoclonal and/or polyclonal antibodies as content (Haab et al., 2001; Macbeath, 2002; Macbeath and Schreiber, 2000). The fact that these reagents had not been designed and selected for the properties that they were expected to display in the antibody microarray applications can to a great extent explain why concerns were raised regarding probe functionality and specificity (Haab et al., 2001; Kingsmore and Patel, 2003; Macbeath, 2002; Mitchell, 2002; Service, 2001; Zhu et al., 2003). Recently, $F(ab')_2$–based microarrays have been fabricated using enzymatically digested monoclonal antibodies as probes, providing an array set-up that may suffer from less non-specific binding (Song et al., 2004).

The issues regarding probe functionality and specificity can, however, be minimized using recombinant antibody libraries designed for microarray applications as probe source (Pavlickova et al., 2004; Wingren and Borrebaeck, 2004; Wingren et al., 2003). In fact, adopting this probe format will, as outlined in Table 1, also address most, if not all, of the inherent limitations associated with the monoclonal and polyclonal antibody probe formats, including availability (number of antibodies, range of specificities), scaling-up (production costs, logistics), molecular properties (wide range of properties, re-design issues) etc. Although monoclonal and polyclonal antibodies play and will continue to play an important role within antibody-based microarray applications, these shortcomings must be considered when setting up the array technology platform (Wingren and Borrebaeck, 2004, 2006). As an example, it has become clear that comparing microarray data generated on different platforms will be challenging (Haab et al., 2005; Wingren and Borrebaeck, unpublished data). Apart from the choice of substrate and analytical principle, the use of probes based on different scaffolds, displaying a range of molecular properties, will be a major factor contributing to such inherent assay variations. To this end, it is not surprising that designed antibody libraries, preferentially created around a single framework, have surfaced as a prominent antibody probe source (Lueking et al., 2005; Pavlickova et al., 2004; Wingren and Borrebaeck, 2004, 2005). Recombinant single-chain Fv (scFv) antibodies, microarray adapted by design has been found to perform (e.g., specificity, functionality and stability) excellently in microarray applications (Ingvarsson et al., 2006a; Steinhauer et al., 2002, 2005; Wingren et al. 2003, 2005; Wingren and Borrebaeck, 2004).

In future work, generating (selecting) binders against all proteins in any target proteome (e.g., ProteomeBinders, an EC FP6 Infrastructures Coordination Action) will clearly become a key challenge. Of note, the Swedish Human Proteome Resource (HPR) Center has launched the first full scale effort in generating monospecific polyclonal antibodies against human target proteins using a high-throughput approach based on the cloning and protein expression of Protein Epitope Signature Tags (PrESTs) (Nilsson et al., 2005). These monospecific antibodies will form a most valuable source and provide a scientific basis for

TABLE 1. A COMPARISON OF THE PROPERTIES DISPLAYED BY MONOCLONAL AND RECOMBINANT ANTIBODY PROBE SOURCES INTENDED FOR MICROARRAY APPLICATIONS

Key issues	*Monoclonal antibodies*	*Recombinant antibodies*	*Comments*
1. Availability	+	++++	The number of available monoclonal antibodies is small, meaning that finding existing antibodies with the desired specificities will be a major limitation. Large scFv and Fab recombinant human antibody libraries ($>10^{10}$ members) that will provide access to probes with "any" desired specificity are available (Knappik et al, 2000; Soderlind et al, 2000; www.bioinvent.se; www.cambridgeantibody.com; www.morphosys.com).
2. Scaling-up	+	++++	Producing thousands of monoclonal antibodies will not be logistically and economically feasible, making the process of fabricating high-density arrays very demanding. Access to large recombinant antibody libraries will make the issue of scaling up feasible.
3. Renewability	++++	++++	
4. Choice of scaffold/ fixed scaffold	+		A monoclonal antibody will be based on one of several possible scaffolds (frameworks) selected by the original B cell, while recombinant antibodies can be based on the choice of scaffold selected by the user, e.g. displaying desired on-chip properties. Of note, molecules based on one fixed scaffold will all display very similar properties (e.g. on-chip stability) making them well suited as probes. While recombinant antibody libraries based on a single scaffold are available (Soderlind et al, 2000), monoclonal antibodies are based on many different scaffolds.
5. Re-design of probe	+	++++	While monoclonal antibodies based on different frameworks would have to be optimized individually, all members of a recombinant antibody library, based on a single framework, could be optimized/re-designed simultaneously.

selecting probe members useful in future microarray applications. Thus, adopting high-performing recombinant antibody libraries as probe source will obviously provide a jump start also in this issue. Other efforts, some of which are already under way, includes the design of novel libraries or re-design of already existing libraries to improve even further probe properties such as on-chip stability and functionality as well as affinity-tag design (Wingren and Borrebaeck, 2004). The latter point will be of utmost importance when fabricating self-addressable arrays, an array design that is likely to evolve rapidly during the coming years. In the context of generating binders against the whole proteome, several additional features must also be considered. Firstly, it needs to be discussed whether the probes should be directed against either the (i) native protein (most common today), (ii) in silico defined linear epitopes (e.g., PrESTs), (iii) peptide fragments thereof (e.g., www.epitomebiosystems.com), or (iv) combinations thereof. Adopting for example, the EpiTag technology, specific antibodies against short sequences (EpiTags) are generated. These affinity reagents can then be used to analyse the sample, in the format of enzymatically digested peptides, allowing multiplexed, quantitative assays to be performed (www.epitomebiosystems.com). Secondly, the num-

ber of probes generated against every analyte will have a major impact on the assay design flexibility (e.g., whether or not sandwich approaches can be adopted).

DESIGN AND FABRICATION OF ARRAYS

To date, mainly low- to medium-density antibody microarrays have been fabricated on various solid supports by arraying the probes one by one in the pL scale, using either non-contact (more gentle for biomolecules, less carry-over effects) or contact printers (faster). These traditional designs have worked well, but recent work have shown that several interesting alternative approaches are well under way with respect to both design (low- vs. high-density set-ups, nano vs. micro-arrays, intact protein detection vs peptide detection) and array fabrication (spotted vs. self-addressable arrays, cell-free protein expression vs. probe production/arraying).

Recent advances

The development of high-density microarrays has been hampered due to technical aspects. Based on commercial aspects, the biotech industry have focused more and more on fabricating low-density focused microarrays in order to more rapidly be able to deliver solid products addressing specific needs—and not proteome-wide issues (as initially planned) (Sheridan, 2005). Still, high-density arrays will be required in order to allow us to perform truly proteome-wide analysis. To date, the main reasons for the observed limitation in density include, (i) limited availability of high-quality probes, (ii) limited access to high quality substrates allowing oriented affinity immobilization (of non-purified probes), and iii) technical issues (e.g., speed of the dispenser and spot size).

So far, regular antibody microarrays have been fabricated by dispensing of antibodies on 1-, 2-, or 3-dimensional solid supports frequently resulting in $\leq$200 μm sized spots (Haab, 2003; Kusnezow and Hoheisel, 2003; Macbeath, 2002; Pavlickova et al., 2004; Steinhauer et al., 2005; Wingren and Borrebaeck, 2004; Zhu and Snyder, 2003), clearly illustrating one of the key limitations for scaling up the array densities. However, bionanotechnology may provide us with the new tools required to bypass this limitation (Cheng et al., 2005; Wingren et al. 2004), in the end allowing us to fabricate even mega-dense nanoarrays (>100,000 probes) (Wingren et al., 2004). Angnenedt et al. (2004a) have designed a nanowell array on glass microplates with a well volume of 1.5 μL and a well-to-well distance of 2.25 mm. Reagent volumes as low as the 100 nL range was found to be sufficient for detection (Angenendt et al., 2004a). By adopting a nanoimprint lithography approach, Hoff et al. have fabricated 10-nm-sized dots over large areas (Hoff et al., 2004). As proof-of-concept, the authors managed to generate nanoscale polyclonal antibody patterns that could be used to detect antigen (Hoff et al., 2004). Moreover, a truly nanostructured (vials) surface was fabricated, composed of 7 $\times$ 7 vials arrays ($\geq$60–90 nm in diameters) with a footprint of $\geq$20 $\times$ 20 μm (Bruckbauer et al., 2004). Polyclonal antibodies could be adsorbed in the vials and binding of fluorescently labelled antigen was then readily detected (Bruckbauer et al., 2004). In addition, an attovial-based recombinant antibody array has recently been produced, where vials with diameters in the range of 200 nm to 5 μm (corresponding to well-volumes of 6 attoliter to 4 femtoliter) were generated by electron beam lithography (Ghatnekar-Nilsson et al., 2006). The set-up could be successfully used to detect low-abundant analytes in directly labeled complex proteomes, such as serum. A key step in the fabrication of high-density nanoarrays will be how to functionalize the individual wells with unique probes (Wingren et al., 2004). There are already various technologies available for this purpose, such as nanoimprint lithography, enabling arrays displaying a density up to 160 Gspots/cm^2 to be generated (Demers et al., 2002; Heidari et al., 1999; Hoff et al., 2004). Other interesting approaches to address individual vials include, nanopipetting (Bruckbauer et al., 2004), nanoarraying (e.g. www.bioforcenano.com), as well as micro- and nano-fluidics.

The concept of designing high-density arrays has stimulated the development of novel means of producing antibody as well as protein arrays in general. Firstly, well-based arrays have been developed for cell-free protein expression (and *in situ* immobilization) (Angenendt et al., 2004a; He and Taussig, 2001; www.helixbiopharma.com). In the "protein *in situ* array" (PISA) approach, the array is generated in a one-step procedure directly from PCR-generated DNA fragments by cell-free protein expression and *in situ* im-

mobilization via an affinity tag (e.g., his-tag) at the surface (He and Taussig, 2001). Adopting the heterodimer protein technology, peptide coils are anchored in the bottom of the wells, and plasmids containing peptide coil DNA and protein target DNA, engineered to "synthesize" peptide coil and target protein complex, are added. These complexes (e.g., an antibody) fuse to the anchored peptide coils on the surface of the wells, oriented appropriately (www.helixbiopharma.com). Of note, Ramachandran *et al.* have designed a similar approach, the nucleic acid programmable protein array (NAPA) methodology, for self-assembling protein microarrays that can be produced directly on planar glass slides in a cell free expression system (Ramachandran et al., 2004). An alternative approach has recently been described by Delehanty et al., in which a cellular microarray platform was devised for comparative functional analysis of plasma membrane expressed scFv (Delehanty et al., 2004). Other means of generating self-assembling microarrays could be to use probe functionalized vessels, for example, vesicles (Stamou et al., 2003; Svedhem et al., 2003). Consequently, manners of generating self-addressable protein arrays have recently gained a lot of interest (Ramachandran et al., 2004; Wacker and Niemeyer, 2004; Wacker et al., 2004). Each probe is then equipped with a unique zipcode tag (e.g. short stretch of DNA) that will direct the probes to their unique spot on the chip functionalized with a corresponding matching tag (e.g., complementary stretch of DNA). In the end, the probes can simply be poured onto the chip and they will find the way to their spot on their own. Approaches like this will undoubtedly be the focus of major interest during the coming years, especially when adopting bionanotechnology methodologies.

Traditionally, mainly intact protein analytes have been detected using antibody-based microarrays, but novel array designs for peptide analyte detection have recently been brought forward targeting enzymatically digested protein mixtures (Scrivener et al., 2003; Warren et al., 2004; www.epitomebiosystems.com). Scrivener et al. demonstrated proof-of-concept for short chain antibodies as well as polyclonal antibodies on hydrogel pads, i.e. planar arrays, using MALDI-TOF MS as read-out system (Scrivener et al., 2003). In comparison, Warren et al. have presented a bead-based antibody array design coupled to tandem MALDI-TOF MS detection (Warren et al., 2004). The latter set-up was shown to provide sensitive (fmol levels), multiplex, and quantitative measurements, simultaneously allowing the user to identify the protein(s). Based on the EpiTag technology, a sensitive and quantitative planar array platform, using sandwich and/or competitive assay designs for read-out, have been developed (www.epitomebiosystems.com). By addressing peptides instead of intact protein analytes, a quantitative label-free platform may be devised, thereby eliminating some of the key concerns within the traditional protein array set-ups. In addition, such set-ups may also significantly reduce the number of probes required to target an entire proteome, if antibodies specific for peptides unique for a set of proteins rather than a single protein are applied. Moreover, the awareness of the peptidome for reflecting biological events and for disease diagnostics have also increased significantly during the last few years (Liotta and Petricoin, 2006; Schulte et al., 2005), clearly highlighting the potential impact of these new array designs.

SAMPLE FORMAT

The format, availability, complexity and dynamic nature of a proteome represent major technological challenges, not only for antibody-based microarrays, but for any proteomic technological research platform (Kingsmore, 2006; Wingren and Borrebaeck, 2004). While major efforts have been placed upon developing the basic antibody (protein) array technology, little attention has so far been placed upon the sample itself.

Recent advances

All samples generated in a soluble format, whether in a native, denatured or digested format, can potentially be analyzed by antibody-based microarrays (Wingren and Borrebaeck, 2004). While a majority of the antibody array platforms at hand target water-soluble proteins, the first designs based on monoclonal antibodies (Belov et al., 2001, 2003; Ellmark et al., 2006a; Ko et al. 2005a,b) or recombinant scFv antibodies (Dexlin et al., 2006) targeting cell-surface membrane proteins in the format of intact cells have only recently been presented. In this context, it should be noted that the cell membrane proteome plays a key role.

First, membrane proteins constitute an extremely important group of proteins being one of the most common targets for disease diagnostics, biomarker discovery and therapeutic drugs (Jang and Hanash, 2003). Second, characterization of the changes that occur in the cell surface membrane proteome during differentiation in response to various stimuli (e.g., activation) will provide an increased understanding of fundamental processes in both health and disease. In comparison, there are very few effective classical strategies for high-throughput profiling of cell surface membrane proteomes (Jang and Hanash, 2003). Protein tagging of intact cells followed by 2-dimensional gel analysis has often been used (Jang and Hanash, 2003), and additional techniques, such as ELISA and FACS, can also be adopted, but only for low-throughput approaches. Thus, antibody-based microarrays may provide the novel tools required in order to perform high-throughput cell membrane proteomics in a facile manner.

The reduced sample consumption (sub μl scale) in the microarray format is essential as only minute volumes of precious (clinical) samples are often available; volumes in the pL scale may in fact be sufficient if the sample is arrayed as well, using conventional spotters (Angenendt et al., 2004b; Borrebaeck et al., 2001; Wingren et al. 2005). As for any proteomic approach, the way the sample is collected will also play a major role (Haab et al., 2005; Wilkins et al., 2006). In a recent study by Haab et al. (2005), the impact of sampling methods was investigated by analyzing serum and plasma samples collected using various anticoagulants. These studies showed that EDTA-plasma was the preferred choice when collecting blood samples.

Furthermore, the sample complexity is a key feature that may impair the analysis by (i) making it difficult to label the samples in a representative manner, and/or by (ii) causing high non-specific binding and thereby significantly reducing the assay sensitivity. In traditional proteomics, the use of various pre-fractionation strategies and/or removal of high-abundant proteins to reduce sample complexity is often critical in order to perform the analysis in an adequate manner (Adkins et al., 2002; Pieper et al., 2003; Tirumalai et al., 2003). One problem associated with any protein separation/depletion technique is, however, that low abundant proteins may be removed along with the high abundant species (Adkins et al., 2002; Pieper et al., 2003). In the case of antibody microarrays, one recent study have shown that a simple one-step fractionation (based on size) of a proteome considerably enhanced the detection of low molecular weight (<50 kDa), low-abundant (sub-pg/ml range) protein analytes (Ingvarsson et al., 2006a). While the development and implementation of (improved) fractionation procedures may thus provide one avenue for designing antibody arrays for proteome analysis, the main efforts strive to develop technology platforms capable of handling complete, i.e. non-fractionated, proteomes (Wingren and Borrebaeck, 2004, 2006). By optimizing the sample labeling protocol as well as the assay protocol (e.g., blocking, washing, and sample buffers, choice of substrate), these efforts have shown that high-performing antibody microarray platforms capable of targeting not only high- to medium-abundant analytes (e.g., Gao et al., 2005; Knezevic et al., 2001; Miller et al., 2003; Orchekowski et al., 2005; Sanchez-Carbayo et al., 2006; Sreekumar et al., 2001), but also low-abundant analytes (e.g., Ellmark et al., 2006b; Ingvarsson et al., 2006a,b; Wingren et al., 2006) in directly labeled proteomes can be achieved.

DETECTION

To date, a majority of all antibody microarray technology platforms have relied on fluorescence-based read-out systems (for review see e.g. Wingren and Borrebaeck, 2004). A variety of set-ups, in which the sample has been directly labeled with a fluorescent tag (e.g., Cy-dyes, Alexa-dyes or ULS labels) (www.gehealthcare.com; www.kreatech.com; www.probes.invitrogen.com) or a hapten (e.g., biotin) (e.g., www.kreatech.com; www.piercenet.com) have been devised. In addition, the potential use of fluorescent dye-doped particles, Rubpy encapsulated in silica matrix particles, functionalized with the secondary reagent (e.g., streptavidin) have also been indicated to provide an alternative way of reaching ultrasensitive detection (Lian et al., 2004). A limit of detection (LOD) in the nM to fM range (zeptomole range) have commonly been reported, even for complex samples such as human whole serum (e.g., Beator, 2002; Haab et al., 2001; Ingvarsson et al., 2006a,b; Kusnezow and Hoheisel, 2002; Macbeath and Screiber, 2000; Pawlak et al., 2003; Schweitzer et al., 2000; Sreekumar et al., 2001; Wingren et al., 2005, 2006). Hence, the LODs

observed are already within the suggested range (attomole level) required to perform well within clinical proteomics (Kusnezow and Hoheisel, 2002). The LOD may be even further improved by adopting various signal amplification techniques readily available, such as rolling circle amplification (RCA) (Kingsmore and Patel, 2003; Schweitzer et al., 2002; Zhou et al., 2004) or thyramide signaling amplification kits (www.perkinelmer.com). A variety of scanners, based on non-confocal, confocal or planar wave guide technology [see e.g. www.arrayit.com; www.perkinelmer.com; www.zeptozens.com), can then be used for reading the microarrays.

Recent advances

A major advance, as outlined above, is the observation that antibody microarray technology platforms can be designed to display a sensitivity in the sub-pg/ml range whether directly labeled pure analytes or complex proteomes are targeted (Ingvarsson et al., 2006a, 2006b; Wingren and Borrebaeck, 2004; Wingren et al., 2005, 2006). Thus, low abundant (clinically relevant) analytes can be addressed. In combination with the prospect of adopting current as well as novel signal amplification strategies, e.g., the proximity ligation methodology (Fredriksson et al., 2002), the sensitivity should thus no longer be a major issue.

In the past, efforts have been made to incorporate sandwich approaches, which in the end may enhance the sensitivity and specificity of the set-ups even further (Beator, 2002; Kusnezow and Hoheisel, 2002; Pawlak et al., 2003; Schweitzer et al., 2000). The sandwich approach works well as long as small focused arrays are constructed. However, the logistic issues involved with scaling up such platforms combined with the observation that a threshold of about 50 probes per sandwich array might be the upper limit to still maintain adequate assay features (Haab, 2003), makes it a less attractive option when designing high-density microarrays. However, a novel array design, the double-chip antibody (protein) arrays—force-based multiplex sandwich assays—may provide one solution to this problem (Blank et al., 2004; Gilbert et al., 2003, 2004). In this set-up, a second chip surface is used for the local application of detection antibodies, thereby efficiently reducing any potential antibody cross-reactivities.

The choice of analytical principle, and in particular the issue regarding label-dependent vs label-free detection method has gained significant interest in the last years (for reviews see Cheng et al., 2005; Espina et al., 2004; Pavlickova et al., 2004; Ramachandran et al., 2005; Tomizaki et al., 2005; Wingren and Borrebaeck, 2004; Zhu and Snyder, 2003). In fact, within the field of protein microarrays as a whole, there is a major interest in adopting label-free detection methods to eliminate problems associated with protein labeling. Here, we will focus mainly on the efforts involving antibody arrays.

First, MS is an appealing analytical principle as it may enable the user not only to detect but also to identify the bound ligand. SELDI-TOF MS has, for example, been implemented, providing 8 or 16 spots arrays displaying a wide variety of surface chemistries (www.ciphergen.com). Adopting MALDI-TOF MS, proof-of-concept studies has been generated for recombinant scFv on silicon-based substrate (Borrebaeck et al., 2001), and monoclonal antibodies on either a 3-dimensional methacrylate polymer-based substrate (Gavin et al., 2005), planar porous silicon surface (also compatible with fluorescent read-out (Finnskog et al., 2004), or porous silicon nanovials (Finnskog et al., 2006). While a sensitivity in the attomole- (Borrebaeck et al., 2001) to zeptomole-range (Finnskog et al., 2004) may be achieved, critical issues regarding, in particular, blocking and sample complexity remains to be resolved (Wingren and Borrebaeck, unpublished observations).

Second, surface plasmon resonance (SPR) and variants thereof, e.g., localized SPR (LSPR), have also gain significant interest as a label-free detection principle, simultaneously providing unique binding characteristics data (e.g., Dahlin et al., 2005; Usui-Aoki et al., 2005; www.biacore.com; www.genoptics-spr.com; www.graffinity.com). The different platforms display great potential, although outstanding issues regarding sensitivity, number of spots and dynamic range have to be addressed.

Third, by adopting nanotechnology (for review see e.g., Cheng et al., 2005), a series of methodologies may become available, so far including atomic force microscopy (also compatible with fluorescent read-out) (Lynch et al., 2004), and nanomechanical cantilevers (Arntz et al., 2003; Backmann et al., 2005; Dutta et al., 2003; Ji et al., 2004; Weeks et al., 2003). Proof-of-concept experiments has been generated for both intact antibodies (LOD of 20 μg/ml) [e.g., Arntz et al., 2003] as well as recombinant scFv (LOD of 1 nM) [e.g., Backmann et al., 2005].

Fourth, other potential methodologies could include label-free detection by scanning Kelvin nanoprobe (Thompson et al., 2005), piezo-based immunosensors (shen et al., 2005), and quart crystal microbalance with dissipation monitoring (QCM-D) (LOD in the pM range) (Larsson et al., 2005). The latter methodology will, in analogy to SPR, also provide opportunities to follow the binding in real-time thus providing data about the kinetics.

Taken together, all of these non-label dependent approaches are promising, but have so far only been used for small prospective arrays. In the end, more developmental work will be required before any of these methods could become generally available.

Finally, a series of recent papers have clearly highlighted the crucial impact of masstransport on assay kinetics and sensitivity when working in the microarray format (Klenin et al., 2005; Kusnezow et al., 2005, 2006). This involves migration of the analyte both in solution and across the surface of the solid support. The authors concluded that this issue is not sufficiently addressed in the design of many set-ups, outlining novel avenues for additional technology improvements.

MICROARRAY DATA

The field of antibody (protein) microarray have developed at a rapid rate during the last few years. While a major focus has been placed upon the technology platform (e.g., probes, surface, detection, etc.), similar efforts focused towards the downstream processes, data handling, bioinformatics, and data reporting etc remains to be launched (Wilkins et al., 2006). In comparison, the more mature field of DNA microarrays have established guidelines for data reporting. In this case, data compliant with MIAME—minimum information about a microarray—and deposited at public repositories are required for publication [MIAME hemsida]. It is probably only a matter of time before the field of protein microarrays adopts similar standards.

Recent advances

Regarding protein microarray data quantification/normalization/handling etc, no standardized procedures yet exist. Still, validated procedures and softwares have in many cases been more or less directly adopted from the DNA microarray community. The first publications, addressing standardization of antibody (protein) microarrays, have recently been published (Hamelinck et al., 2005; Olle et al., 2005; Perlee et al., 2004). Perlee and co-workers have taken the first steps towards development and adoption of standards that in the end could (more readily) permit platform comparisons and benchmarking (Perlee et al., 2004). Olle *et al.* have designed an internally controlled antibody microarray (Olle et al., 2005). In this set-up, one colour detected represents the amount of antibody spotted, and the other the amount of analyte bound. The signal from the amount of antibody spotted was then used to normalize the data, resulting in decreased variability and lowered limit of detection. In the paper by Hamelinck and co-workers, the authors described their work on optimizing the normalization of antibody microarray data to enable comparison of chip-to-chip data (Hamelinck et al., 2005). They tested and compared seven different normalization methods by their effects on reproducibility, accuracy and trends in data set. They found normalization with ELISA determined data of a selected analyte present in the sample or against a protein spike-in of known concentration to be the best approaches. Interestingly, not all ELISA determined analytes worked well for normalization, indicating on analyte-specific problems. Similarly, we have found protein spike-ins to work well for antibody microarray data normalization (Ingvarsson et al., 2006b). In comparison, a set of house-keeping genes are commonly used to normalize DNA microarray data. Although similar house-keeping proteins may be difficult to find for antibody/protein microarrays, we have recently shown that such "global" normalization against a given set of protein analytes anticipated to display no or very small inter-sample variations worked very well (Ingvarsson, Wingren, and Borrebaeck, unpublished data). The issue of data handling/normalization and bioinformatics is expected to grow exponentially as the field of antibody (protein) microarrays continues to mature towards more high-density arrays combined with (more) clinical applications.

CONCLUSION

Antibody-based microarray is a novel technology that holds great promise within proteomics. As outlined in this review, the technology has evolved significantly during the last few years, and the first high-performing antibody microarray technology platforms have started to emerge. In parallel, the numbers of applications in which antibody-based microarrays have been applied have exploded during the very last years, a trend that is expected to continue in the coming years. As described (Gao et al., 2005; Knezevic et al., 2001; Miller et al., 2003; Orchekowski et al., 2005; Sanchez-Carbayo et al., 2006; Sreekumar et al., 2001) and reviewed elsewhere (Borrebaeck, 2006; Kingsmore, 2006; Wingren and Borrebaeck, 2004), various applications have already been developed within disease diagnostics, screening, and protein expression profiling, ranging from focused assays to semi-proteome analysis. The potential and recent advances of the technology within oncoproteomics is of particular interest (Borrebaeck, 2006; Wingren and Borrebaeck, 2004). Intense efforts are currently under way to address the remaining key technological issues (Wingren and Borrebaeck, 2006) that will take the technology to the next level and truly provide a multiplex, sensitive and high-throughput proteomic research tool. Antibody-based microarrays will then be one among the most prominent technologies available for cutting edge research within the field of (disease) proteomics.

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